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13. ABSTRACT *(Maximum 200 Words)*

The risk of developing breast cancer rises with obesity, however the cause of this phenomenon is unknown. An intriguing candidate is leptin, a protein produced almost exclusively by adipocytes, a prominent stromal component in mammary tissue. The studies of this proposal were designed to test the hypothesis that the interaction of leptin with its receptor regulates normal and pathologic mammary epithelial cell proliferation and/or differentiation. As part of my training, I have demonstrated that leptin prevents growth factor induced cellular proliferation and DNA synthesis. I hypothesize that leptin may force mammary epithelial cells from a proliferation to a differentiation pathway. However, the observed responses were small and difficult to analyze. This necessitated the development of an in vitro culture system that would more accurately determine the functional consequences of leptin action through the leptin receptors. The culture of HC11 mammary epithelial cells on a collagen matrix induces the expression of leptin receptor long mRNA. However, upon addition of insulin/epidermal growth factor this expression is lost. More work is necessary to refine this system to accurately portray the consequences of leptin effects on mammary epithelial cells and is currently in progress. Complementing my research training has been my participation in meetings and manuscript/grant submissions.

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4. Introduction

The risk of developing breast cancer rises with obesity, however the cause of this phenomenon is unknown. The growing adipocyte population during obesity may contribute directly to breast cancer by providing an excess of growth factor(s) that are required for normal mammary epithelial cell proliferation. An intriguing candidate is leptin, a protein produced almost exclusively by adipocytes for the regulation of energy metabolism. Leptin also influences reproductive development. As lactation is an extension of reproduction, the premise is established for leptin to participate in mammary gland development. Preliminary evidence in our lab indicated that mammary epithelial cells express leptin receptors and may be capable of responding to leptin released by the surrounding adipocytes. **The studies of this proposal were designed to test the hypothesis that the interaction of leptin with its receptor regulates normal and pathologic mammary epithelial cell proliferation and/or differentiation.** Aim 1 will examine leptin's role in mammary gland development by assessing the mRNA and protein expression of leptin and its receptor in the mammary glands of virgin, pregnant, and lactating mice. Archival human breast tissue samples collected from healthy and breast cancer patients also will be examined to determine if leptin or leptin receptor expression changes with tumor development. Biochemical, immunohistochemical, molecular techniques will be used to qualify mRNA and protein expression. Aim 2 will evaluate if the interaction of leptin with its receptor induces proliferation and/or differentiation of normal and tumor-derived mammary epithelial cells. The first objective of this aim will judge the proliferative effects induced by leptin-containing pellets implanted in the mammary glands of leptin-deficient (*ob/ob*) mice. The second objective will complement the *in vivo* studies by examining the direct effects of leptin administration on the proliferation and differentiation of normal and tumor derived mammary epithelial cells. The final objective will examine the biochemical signaling mechanisms by which leptin activates epithelial cell responses by determining the activation of specific signal transducers and activators of transcription (STAT) proteins through electrophoretic mobility shift and supershift assays. Understanding the functional role of adipocyte-derived factors on mammary cell growth is imperative, especially because of the increased risk of breast cancer that occurs with obesity. The results of these studies will contribute directly to the knowledge of mammary gland development and possibly tumor development by providing new information regarding the signaling pathways between the adipocyte-rich stroma and mammary epithelial cells.

5. Body

The first year of this award was to describe leptin and leptin receptor mRNA and protein expression during normal post-natal mammary gland development in the mouse and by various breast tumors collected from patients at the University of Oklahoma Health Sciences Center and embedded in paraffin. A series of papers that were published following the submission and the subsequent awarding of this training grant induced me to alter my proposed series of goals. One of these papers detailed the expression of mRNA for both long and short forms of leptin receptor mRNA during pregnancy, with the highest expression at mid-pregnancy and the lowest at lactation in the mammary epithelial cells collected from sheep [1]. This differed from leptin mRNA levels that appeared to decrease from pre-pregnancy through lactation in mice. However, leptin protein expression did not coincide with mRNA levels in that leptin protein in milk that increased dramatically just prior to parturition and was attributed to the maternal blood supply [2]. A separate study also detailed the expression of leptin mRNA in several mammary epithelial cell lines, as well as leptin protein in breast tumor homogenates [3]. The information from these studies had already initiated the descriptive work that I had intended to conduct first. Therefore, I decided to pursue the mechanistic work that would delineate the functional consequence of leptin/leptin receptor interactions in mammary epithelial cells.

5.1 Materials and Methods

Reagents. Reagents were purchased from Sigma (St. Louis, MO) unless otherwise specified. Fetal calf serum was stripped with dextran-coated charcoal prior to use in cell culture. [Methyl-^{3}H] thymidine was purchased from ICN Pharmaceuticals, Inc (Costa Mesa, CA). The HC11 mammary epithelial cell line was originally provided by Dr. D. Medina (Department of Cell Biology, Baylor College of Medicine, Texas Medical Center, USA). Serum free media (**SFM**) consisted of phenol-red free DME/F12 supplemented with sodium bicarbonate (3.7 mg/ml), bovine serum albumin (0.1 mg/ml), transferrin (10 μg/ml), sodium pyruvate (0.1 mM), gentomycin (50 μg/ml), penicillin (5 U/ml), and streptomycin (5 μg/ml). The HC11 cells were cultured in SFM supplemented with 5% charcoal-stripped fetal calf serum.

DNA synthesis. HC11 cells resuspended in SFM plus 5% FCS were seeded at 5000 cells/well in a 96 well plate and allowed to adhere for 2-3 hours prior to removing media. The cells then were serum starved overnight at 37°C, 5% CO_2 prior to adding increasing concentrations of murine leptin (0, 1, 10, 100 ng/ml). At 30 hours, 0.5 μCi ^{3}H thymidine was added per well and incubated an additional 18 hours for a total of 48 hours. To determine thymidine incorporation, the cells were rinsed twice with phosphate buffered saline, fixed with ice-cold 5% trichloroacetic acid for 2 hours at 4°C, rinsed twice with cold 5% trichloroacetic acid, and then solubilized with 0.5 N sodium hydroxide and 0.1% Triton-X 100. This solution then was transferred to scintillation vials containing 2 ml scintillation fluid and counted.

Cell proliferation. HC11 cells were treated as outlined above for DNA synthesis. In place of 3H-thymidine, 25 μl/ well of 1 mg/ml MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was added at 46 hours and incubated for an additional 2 hours at 37°C. A 100 μl aliquot of extraction buffer (20% lauryl sulfate dissolved in 50% dimethyl formamide, pH 4.7) was added to each well, incubated overnight to allow breakdown of crystals, and the absorbance read at 490 nm on a Titertek plate reader. Live cells convert the yellow MTT die to purple formazan through mitochondrial oxidation. The number of live cells was determined by comparison to a standard curve generated from a known quantity of HC11 cells generated from the same passage.

RT-PCR. Total RNA was isolated by the guanidinium thiocyanate method outlined by Chomczynski and Sacchi [4]. Prior to reverse transcription, 2.5 μg total RNA was resuspended in a solution of 1x MMLV buffer, 40 units RNasin, and 2 U/ml DNase and incubated at 37°C for 20 min, followed by heat inactivation at 75°C for 5 min. Reverse transcription was initiated by addition of 40 units RNasin, 25 pmol oligo dT15, 1 mM each dNTP, and 50 units MMLV-RT prior to incubation at 42°C for 45 min. Five μl (0.25 μg original RNA) then was subjected to PCR amplification of cDNA sequences specific for leptin, leptin receptor long (b), leptin receptor short (a), and β-actin. The primer sequences and product sizes can be seen in Table 1. The cDNA was subjected to either 20 or 35 cycles (all but β-actin) of 1 min denaturation at 94°C, 1 min annealing at either 60°C (all but leptin) or 65°C, and 1 min extension at 72°C. The products were separated in a 1.5% agarose gel and visualized by excitation of ethidium bromide.

Table 1. Primer sequences and predicted product sizes.

Primer		Sequence	Size	Reference
Murine leptin	for	5'-agt gcc tat cca gaa agt cca gga	417	[5]
	rev	5'-ctg ttg aag aat gtc ctg cag aga		
Murine leptin receptor a (short)	for	5'-aca gtt ctg gct gtc aat tcc c	611	[6]
	rev	5'-gta tgg act gtt ggg aag ttg g		
Murine leptin receptor b (long)	for	5'-aca ctg tta att tca cac cag ag	445	[7]
	rev	5'-tgg ata aac cct tgc tct tca		
β-actin	for	5'-cct aag gcc aac cgt gaa aag	600	[8]
	rev	5'-tct tca tgg tgc tag gag cca		

5.2 Results and Discussion

DNA synthesis and cell proliferation. Increasing doses of leptin alone did not appear to influence either cell proliferation or DNA synthesis following 48 hours of treatment. However, leptin did halve cell number in most cultures receiving insulin (50 ng/ml), EGF (10 ng/ml), or insulin/EGF when compared to those that did not receive leptin. This appeared to be an all or none effect, as cell number did not vary with leptin dose (Figure 1). This suggests that leptin either blocks cellular proliferation or induces cellular differentiation or apoptosis. In order to better define the consequences of leptin on cell function DNA synthesis was measured following incubation of cells with growth factors +/- leptin. Increasing doses of leptin alone did not alter DNA synthesis by HC11 cells (Figure 1). Similar results were obtained when cells received leptin in conjunction with either insulin (50 ng/ml) or EGF (10 ng/ml). A combination of 1 ng/ml leptin with insulin and EGF almost halved ^{3}H-thymidine incorporation relative to those that did not receive leptin, even though greater leptin doses had no effect. Initially this may appear to contradict the cell proliferation results that demonstrated a reduction in cell numbers by all leptin concentrations, regardless of growth factor received. However it is possible that even though fewer cells are present, the remaining cells may be undergoing differentiation and are in the process of synthesizing DNA for this state. Hence, the higher doses of leptin may cause a greater number of cells to differentiate than lower leptin doses and may cause a differential induction of dna synthesis and subsequent ^{3}H thymidine incorporation.

The small changes observed in the DNA synthesis and proliferation assays were troubling however, and would prevent definitive studies regarding the mechanisms of leptin receptor signaling and subsequent cellular function. Personal communication with Christian Bjorbaek, a member of Jeff Flier's group at Harvard who has actively pursued leptin signaling, stated that this was not unusual and that a suitable in vitro system that does not involve transfection of leptin receptors is not available currently. He recommended that I seriously

consider an in vivo system. However, the monies available in the laboratory are limited. I therefore decided to focus on developing an in vitro system that more closely mimics the in vivo situation. I experienced several difficulties in developing this system - mostly concerning collagen preparation and subsequent RNA extraction. I recently was able to force expression of both the long and short forms of the leptin receptor mRNA in HC11 cells cultured on a collagen matrix with or without insulin (10 μg/ml) and EGF (10 ng/ml) for 5 days (Figure 3). I hope to further refine this system so that I can maximize the cellular responses to leptin without resorting to transfection of leptin receptors. This offers a more realistic approach and may provide the only means of examining "natural" leptin receptor function in vitro.

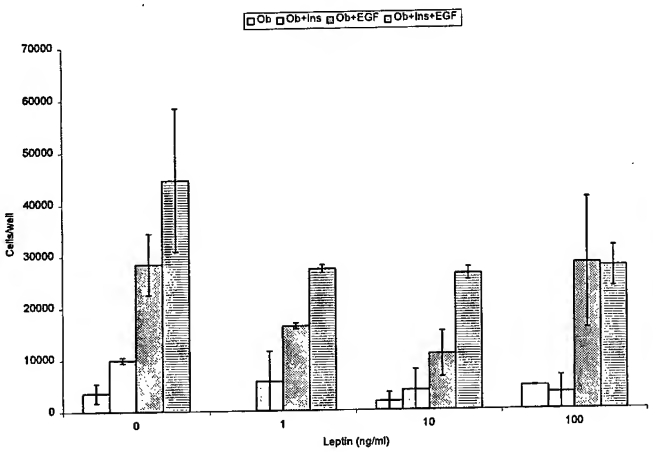

Figure 1. The effects of increasing leptin dose on HC11 proliferation when either in the absence or presence of growth factors: insulin (50 ng/ml), EGF (10 ng/ml), or insulin + EGF. Cell numbers are expressed as the mean +/- SE.

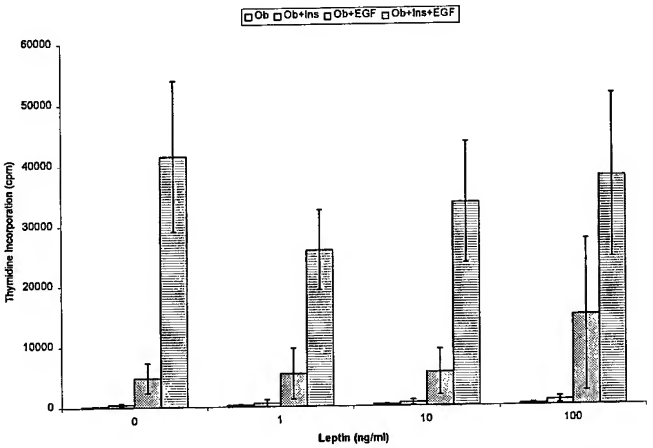

Figure 2. The effects of increasing leptin dose on HC11 DNA synthesis when either in the absence or presence of growth factors: insulin (50 ng/ml), EGF (10 ng/ml), or insulin + EGF. DNA synthesis is expressed as the mean cpm +/- SE.

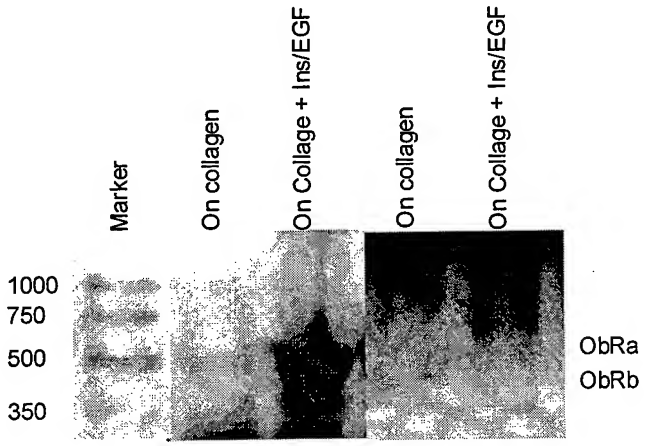

Figure 3. In vitro culture of HC11 cells on collagen either in the presence or absence of insulin (10 μg/ml) and EGF (10 ng/ml). RT-PCR amplification of leptin receptor short (ObRa) and long (ObRb) for 35 cycles.

7. Appendix

7.1 Key research accomplishments

- Establishment of protocols for RT-PCR and immunohistochemistry of leptin and leptin receptor
- Development of an in vitro 3-dimensional culture system for maximal leptin responses
- Leptin reduces proliferation/dna synthesis of a mammary epithelial cell line

7.2 Reportable Outcomes

Manuscripts, Abstracts, and Presentations

Pighetti, GM, W Novosad, C Nicholson, DC Hitt, C Hansens, AB Hollingsworth, ML Lerner, D Brackett, SA Lightfoot, JM Gimble. 2000. Therapeutic treatment of DMBA-induced mammary tumors with PPAR ligands. Anticancer Res submitted.

*Oral presentation entitled "Leptin regulation of mammary cell growth" at the Wilderness Conference XVII, mini-Gordon Conference. Cedar Run, PA, September 1999.

Poster presentation entitled "Peroxisome proliferator activated receptors in rodent mammary carcinogenesis " at the Era of Hope Meeting, Department of Defense Breast Cancer Program, Atlanta, GA, June 2000.

*This presentation represents research directly related to the training grant "Leptin regulation of mammary cell growth" DAMD17-99-1-9344. The other reportable outcomes contributed to my training as a research scientist, but are related to additional projects that I have participated in. Those projects were supported by the U.S. Army Medical and Materiel Command under DAMD17-96-1-6281.

Employment Opportunities Applied For

Assistant/Associate Professor of Animal Science (tenure-track), Department of Animal Science, The University of Tennessee, Knoxville.

Assistant Professor in Lactation Physiology/Mammary Gland Biology (tenure-track), Department of Animal Science, University of Vermont, Burlington, VT.

8.0 Conclusions

In summary, I have demonstrated that leptin prevents growth factor induced cellular proliferation and DNA synthesis. I hypothesize that leptin may force mammary epithelial cells from a proliferation to a differentiation pathway. However, the observed responses were small and difficult to analyze. This necessitated the development of an in vitro culture system that would more accurately determine the functional consequences of leptin action through the leptin receptors. Overall, I had hoped to be further along in these studies and am disappointed that they have not progressed as I well as expected. I recognize that part of these difficulties involve the transition from my former lab at The University of Oklahoma to my current lab at The Pennsylvania State University. The environment, reagents, and equipment differed between the two places and resulted in a re-establishment of protocols that took several months. I also experienced difficulties establishing the three dimensional in vitro culture system. During these

periods I was given the opportunity to participate in other projects in my current and former labs. I have recently sent a manuscript to Anticancer Research and am in the process of preparing 2 additional manuscripts. I also have contributed significantly to the preparation of both NIH and USDA grants in my current lab. I believe the best training is yet to come as the principal investigator of my current lab, Craig Baumrucker, is on sabbatical and I will be responsible for the laboratory in his absence.

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